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C6 ceramide sensitizes pemetrexed-induced apoptosis and cytotoxicity in osteosarcoma cells



Xinhui Zhu^{a,*}, Xueping Du^{b,1}, Xiaolong Deng^c, Hong Yi^a, Shengyu Cui^a, Wei Liu^a, Aiguo Shen^c, Zhiming Cui^a

^a Department of Orthopaedics, The Second Affiliated Hospital of Nantong University, Nantong 226001, People's Republic of China

^b Department of Orthopaedics, Qi-Dong Hospital of Traditional Chinese Medicine, Nantong 226200, People's Republic of China

^c Department of Immunology, Medical College, Nantong University, Nantong 226001, People's Republic of China

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ABSTRACT

Chemotherapy has significantly improved the prognosis of high-grade osteosarcoma (OS), but over 30% of OS patients can still not be cured. Pemetrexed, the newly-developed anti-folate chemotherapy drug, exerted lower efficacy against OS cells. Here, we aimed to increase pemetrexed efficiency, and found that the cell-permeable short-chain ceramide (C6) significantly enhanced pemetrexed-induced viability reduction and death in cultured OS cell lines (U2OS and MG-63). Pemetrexed induced moderate apoptosis in OS cells, which was dramatically augmented by C6 ceramide. The apoptosis inhibitor z-VAD-fmk largely inhibited C6 ceramide plus pemetrexed-induced cytotoxicity and apoptosis in OS cells. By using pharmacological and siRNA-knockdown strategies, we showed that Akt–mammalian TOR (mTOR) over-activation was an important pemetrexed resistance factor in OS cells, and C6 ceramide-mediated pemetrexed sensitization effect was mediated, at least in part, by Akt–mTOR inhibition. Finally, we found that Akt–S6 Kinase 1 (S6K1, an indicator of mTOR activation) was over-activated in human OS tissues. On the other hand, the osteoblastic MC3T3-E1 cells, which expressed lower Akt–S6K1 phosphorylation, were resistant to pemetrexed and/or C6 ceramide. Together, we conclude that C6 ceramide sensitizes pemetrexed-induced apoptosis and cytotoxicity in OS cells probably through in-activation of Akt–mTOR signaling.

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1. Introduction

Osteosarcoma (OS) is one common primary malignancy in children or young adults [1]. An effective treatment strategy for relapsed patients needs to be defined [1–3]. Chemotherapy has significantly improved the prognosis of patients with high-grade OS. However, more than 30% of patients still can't be cured by current treatment regimens [1–4]. As such, there is an urgent need for the development of novel chemotherapeutic agents for OS patients [1,3,4].

Pemetrexed is a novel anti-folate chemotherapy drug. By inhibiting at least three enzymes required in purine and pyrimidine synthesis including thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycylamide ribonucleotide formyltransferase (GARFT), pemetrexed prevents the formation of purine and pyrimidine, which are essential for DNA/RNA synthesis and cancer cell growth [5,6]. Pemetrexed is currently approved for non small cell lung cancer and malignant pleural mesothelioma treatment [7,8]. However, studies have demonstrated that pemetrexed only exerted lower efficacy against OS cell lines [9]. Thus, the aim of this study is to increase the efficiency of pemetrexed in OS cells by adding a known chemo-sensitizer ceramide [10], and to study the underlying mechanisms.

* Corresponding author. Address: 6 North Hai-er-xiang Road, Nantong 226001, People's Republic of China. Fax: +86 513 85061003.

E-mail address: zhuzhuyeah1981@yeah.net (X. Zhu).

¹ Equal contributors.

Ceramide is a molecular that regulates anti-proliferative responses associated with cell apoptosis, growth arrest, differentiation and senescence in multiple types of human cancer cells [11–13]. Studies have shown cell permeable short-chain ceramide could significantly increase the chemo-sensitivity of many chemodrugs including doxorubicin, taxol, histone deacetylase inhibitor (HDACi) and many others [10,14,15]. In the current study, we found that C6 ceramide sensitized pemetrexed-induced apoptosis and cytotoxicity in cultured OS cells.

2. Material and methods

2.1. Chemical and reagents

Pemetrexed, AZD2014 and perifosine were purchased from Selleck Chemicals (Selleck, Shanghai, China); Z-VAD-fmk was purchased from Calbiochem (San Diego, CA). C6 ceramide was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Anti-Akt, S6 Kinase 1 (S6K1) and tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho(p)-Akt (Ser-473), p-Akt (Thr-308) and p-S6K1 (Thr 389) were purchased from Cell Signaling Technology (Denver, MA).

2.2. Cell culture

Human OS cell lines MG-63 and U2OS were obtained from the Chinese Academy of Science (CAS) Shanghai Biological Institute (Shanghai, China). OS cells were maintained in DMEM (4.5 g/l glucose)/Ham F12 (1:1) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 1 U/mL penicillin G (Sigma–Aldrich, St. Louis, MO), and 1 µg/mL streptomycin (Sigma). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. The osteoblastic MC3T3-E1 cells, purchased from CAS Shanghai Biological Institute, were seeded at 1×10^5 cells/ml into 75-cm² flasks and maintained in α -MEM supplemented with 10% FBS and 1 U/mL penicillin G (Sigma), and 1 µg/mL streptomycin (Sigma). This basic medium was replenished every two days. α -ascorbic acid (50 µg/ml, Sigma) and β -glycerol phosphate (5 mM, Sigma) were added to MC3T3-E1 cells for differentiation [16,17].

2.3. Human OS tissue isolation and analysis

Human OS tissues and their surrounding un-affected normal bone tissues from three OS patients (all male, 6–12 years of age) were obtained at the time of surgery. Fresh tissues were thoroughly washed in PBS containing 1 U/mL penicillin G, 200 units/mL penicillin–streptomycin and 2 mM DTT, and then minced by scalpel into small pieces. Tissues were then lysed using tissue specific lysis buffer (Sigma), and analyzed by Western blots. All patients enrolled provided individual informed consent with institutional review board approval of all protocols. The experiments were conducted in accordance with the principles of Declaration of Helsinki and the NIH Belmont Report.

2.4. Cell viability assay

Cell viability was analyzed by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, 3000 cells were plated per well in 96-well plates, and cells were allowed to adhere overnight. After treatment, cell viability was measured by adding 20 µl of MTT reagent (5 mg/ml, Sigma) to culture medium, and cells were incubated for 2 h. Absorbance was measured on a microplate reader (Bio-Rad, Basel, Switzerland) at 490 nm. Percentage of viability was calculated by dividing treatment results by control results and expressing as a percentage.

2.5. Trypan blue staining of “dead” cells

The number of dead cells (trypan blue positive) after indicated treatment was counted, and cell death percentage was calculated by the number of the trypan blue positive cells divided by the number of total cells.

2.6. Detection of cell apoptosis

Apoptosis was detected by an Annexin-V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA). Cells were plated in 6-well plates and allowed to adhere overnight. After treatment, cells were harvested with trypsin–EDTA and were washed twice with PBS, and then incubated for 15 min with Annexin-V-FITC and Propidium Iodide (PI). Both early (Annexin V⁺/PI[−]) and late (Annexin V⁺/PI⁺) apoptotic cells were sorted by the fluorescence activated cell sorter (FACS) machine (Becton Dickinson FACS Calibur, San Jose, CA). The percentage of apoptotic cells was recorded.

2.7. Western blots

After treatment, cells (1×10^6) were lysed using lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS. Twenty micrograms of protein was then resolved by SDS–PAGE. Proteins were subsequently transferred to Immobilon-P membranes for immunoblotting. Blots were probed with specific antibodies and immuno-reactive proteins were revealed by the enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The band intensity of each blot was quantified by Image J software (NIH) before normalizing with the corresponding loading controls.

2.8. Akt siRNA

Two siRNAs with the nucleotide sequence 5'-GUACUUGAU-GAUGAAUUU-3' (#1 Akt1-siRNA) [18] and 5'-GAGUUUGAGUAC-CUGAAGCUGUU-3' (#2 Akt1-siRNA) [19], corresponding to part of the Akt1 mRNA, and the control scrambled siRNA were synthesized by Shanghai Lu-rui Biotech (Shanghai, China). U2OS were seeded in a 6-well plate at 60% confluence in 0% FBS medium. For transfection, 3.0 µl PLUSTM Reagent (Invitrogen, Carlsbad, CA) was diluted in 90 µl of RNA dilution water (Santa Cruz, CA) for 5 min at room temperature. Then, 0.2/0.1 nmol of targeted Akt1 siRNA or scramble siRNA was added to PLUSTM Reagent and left for 5 min at room temperature. To this was added 3.0 µl of Lipofectamine (Invitrogen) and incubation for another 20 min. Finally, the complex was added to the well containing 1.0 ml of medium (no antibiotics, no FBS) [20]. Cells were then cultured for additional 48 h. SiRNA efficiency was determined by Western blots.

2.9. Statistics analysis

All of the experiments were performed in triplicate and were repeated at least three times. Data were expressed as mean values \pm standard deviations (SD) and were analyzed by Student's *t* test or ANOVA followed by the Tukey's multiple comparison; the level of significance was $p < 0.05$.

3. Results

3.1. C6 ceramide enhances pemetrexed-induced cytotoxicity in OS cells

Pemetrexed only exerted low efficacy against OS cell lines [9]. The aim of this study is to test the effect of ceramide on pemetrexed-mediated anti-OS cell ability. A cell permeable short-chain ceramide (C6) was applied. MTT cell viability assay results in Fig. 1A demonstrated that pemetrexed dose-dependently inhibited U2OS cell viability. However, the effect of pemetrexed was overall gentle with an IC-50 over 100 µM, which are consistent with other reports [9]. Significantly, co-administration with C6 ceramide dramatically enhanced pemetrexed-induced viability reduction, and the IC-50 lowered to around 0.2 µM (Fig. 1A). The similar results

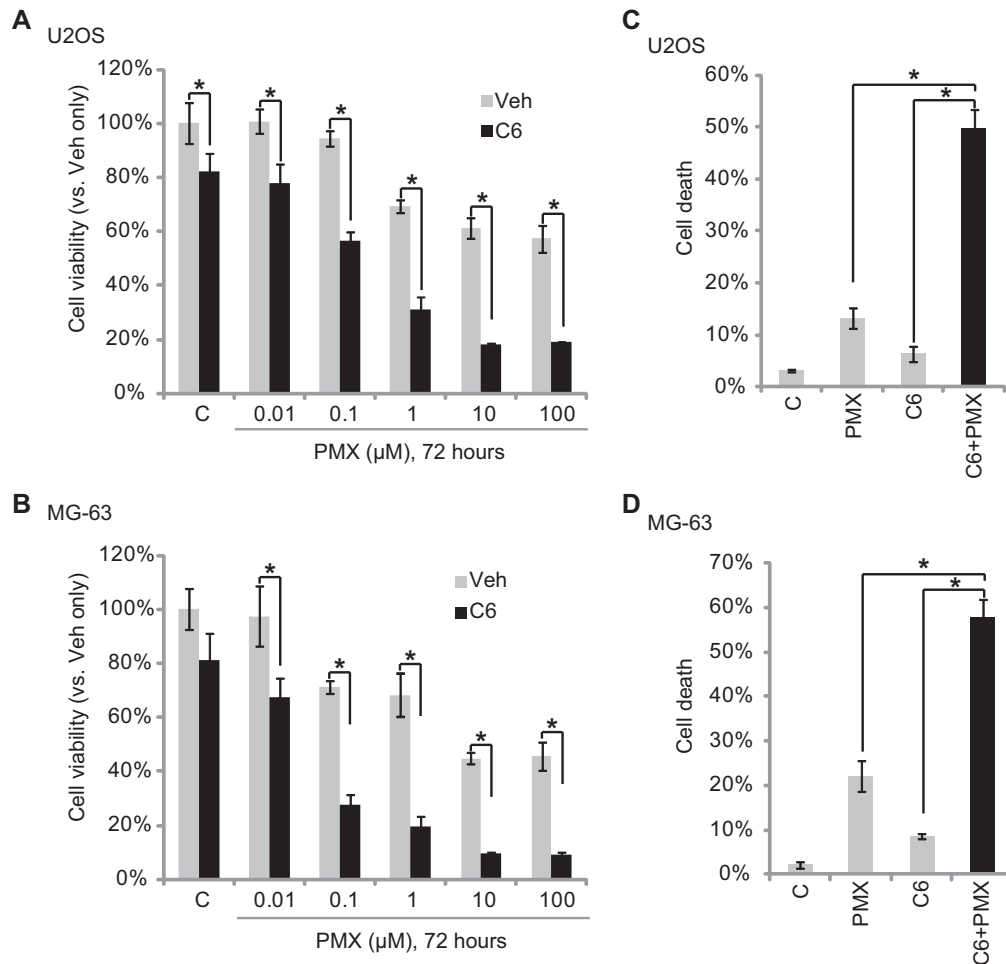


Fig. 1. C6 ceramide enhances pemetrexed-induced cytotoxicity in OS cells. Cell viability of U2OS cells (A) or MG-63 cells (B) after indicated pemetrexed or plus C6 ceramide (C6, 5 μg/ml) treatment was tested by MTT assay. U2OS cells (C) or MG-63 cells (D) were treated with pemetrexed (PMX, 1 μM) and/or C6 ceramide (C6, 5 μg/ml) for 72 h, cell death was analyzed by trypan blue staining assay. Each experiment was performed in triplicate and was repeated at least seven times. "Veh" stands for 0.5% DMSO (A and B). "C" stands for untreated control, **p* < 0.05.

were also observed in another OS cell line (MG-63) (Fig. 1B). Note that, in both U2OS and MG-63 cells, C6 ceramide only slightly inhibited cell viability (less than 15%). C6 ceramide showed most significant sensitization effect with pemetrexed at 1 μM, this concentration was chosen for further experiments. The trypan blue staining assay results in Fig. 1C and D demonstrated that C6 ceramide co-administration dramatically increased pemetrexed (1 μM)-induced OS cell (U2OS and MG-63) death. Together, these results showed that C6 ceramide dramatically enhanced pemetrexed-induced cytotoxicity in OS cells.

3.2. C6 ceramide enhances pemetrexed-induced OS cell apoptosis

Apoptosis is the main contributor of pemetrexed-induced cytotoxicity against a number of cancer cell lines [21,22]. Above results demonstrated that C6 ceramide enhanced pemetrexed-induced cytotoxicity in OS cells, we then examined C6 ceramide/pemetrexed's effect on OS cell apoptosis. Through Annexin V FACS assay, we showed that pemetrexed (1 μM) or C6 ceramide (5 μg/ml) alone only slightly induced apoptosis of U2OS cells and MG-63 cells (5–10%), combination of the two led to a significant increase of cell apoptosis (Fig. 2A and B). Importantly, the apoptosis/general caspase inhibitor z-VAD-fmk [23], which prevented co-administration-induced apoptosis (Fig. 2A and B), largely inhibited OS cell

viability reduction by co-treatment (Fig. 2C and D). Thus, C6 ceramide enhances pemetrexed-induced cytotoxicity through facilitating apoptosis in OS cells.

3.3. C6 ceramide in-activates Akt/mTOR to sensitize pemetrexed in OS cells

Results above showed that C6 ceramide enhanced pemetrexed-induced cytotoxicity and apoptosis in OS cells. Akt/mammalian TOR (mTOR) signaling is an important chemo-resistance factor in OS and other cancers [24]. One main downstream effect of ceramide is Akt in-activation [25]. Thus, we tested the effect of pemetrexed and/or C6 ceramide on Akt/mTOR activation in OS cells. Western blot assay results in Fig. 3A showed that pemetrexed as a single agent had no significant effect on phosphorylation of Akt and S6K1 (an indicator of mTOR activation) in U2OS cells. On the other hand, C6 ceramide, alone or in-combination with pemetrexed, inhibited Akt/S6K1 phosphorylation (Fig. 3A). Interestingly, perifosine, an Akt specific inhibitor [26], as well as a dual mTOR complex 1/2 (mTORC1/2) inhibitor AZD2014 [27] significantly enhanced pemetrexed-induced cell viability decrease (Fig. 3B) and apoptosis (Fig. 3C) in U2OS cells. Further, partial depletion of Akt by targeted siRNAs (two siRNAs with different sequences, Fig. 3D) augmented pemetrexed-induced cytotoxicity in U2OS cells

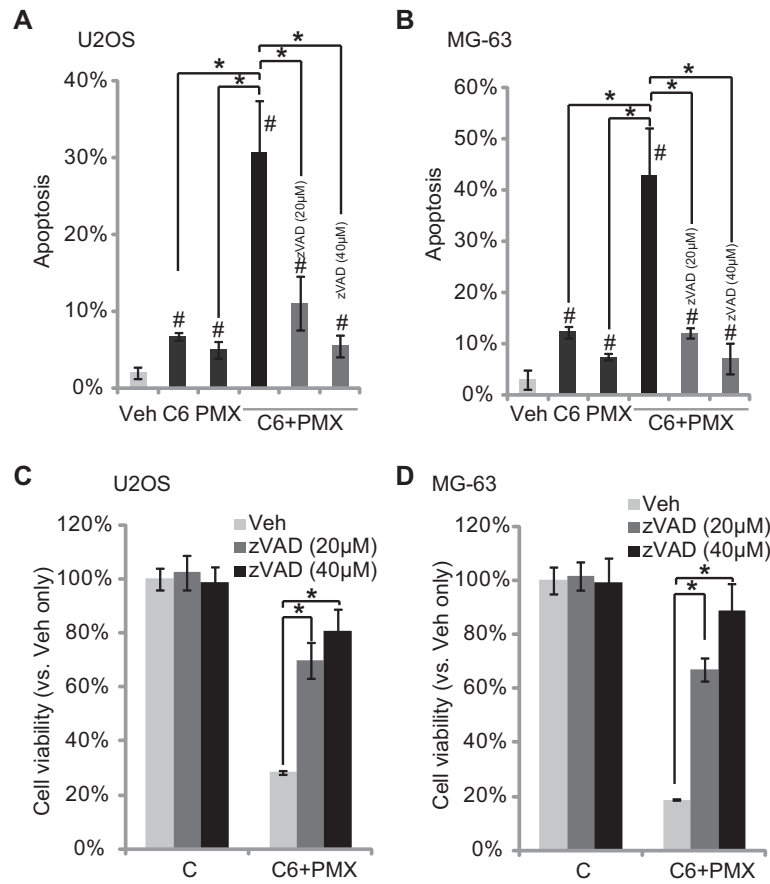


Fig. 2. C6 ceramide enhances pemetrexed-induced OS cell apoptosis. Cell apoptosis of U2OS cells (A) or MG-63 cells (B) after indicated pemetrexed (PMX, 1 μM) and/or C6 ceramide (C6, 5 μg/ml) (48 h), in the presence or absence of z-VAD-fmk (z-VAD, 20/40 μM), was tested by Annexin V/PI FACS sorting. U2OS cells (C) or MG-63 cells (D), pretreated with z-VAD-fmk (20/40 μM), were treated with pemetrexed (1 μM) plus C6 ceramide (5 μg/ml) ("C6 + PMX") for 72 h, cell viability was tested by MTT assay. Each experiment was performed in triplicate and was repeated at least four times. "Veh" stands for 0.5% DMSO. "C" stands for no-pemetrexed treatment. **p* < 0.05, #*p* < 0.05 vs. "Veh" (A and B).

(Fig. 3E and F). Note that S6K1 phosphorylation was inhibited by Akt siRNA-knockdown in U2OS cells, suggesting that Akt is the upstream kinase for S6K1 phosphorylation (Fig. 3D). In MG-63 cells, perifosine and AZD2014 also significantly enhanced pemetrexed-induced cell death and apoptosis (Fig. 3G). Together, these results suggest that Akt-mTOR activation is one main resistance factor of pemetrexed, and C6 ceramide-mediated pemetrexed sensitization effect is mediated, at least in part, by Akt-mTOR in-activation.

3.4. Over-activation of Akt-mTOR in human OS tissues

Above results indicate that Akt-mTOR in-activation is important for C6 ceramide-mediated sensitization effect for pemetrexed. We next tested activation of this pathway in human OS tissues through Western blot assay. As shown in Fig. 4A–C, in all three human OS tissues, Akt/S6K1 phosphorylation was significantly higher than that in surrounding normal osteo-tissues (refer to the quantified data in Fig. 4D). Significantly, osteoblast like MC3T3-E1 cells, where Akt/S6K1 phosphorylation was lower (Fig. 4E), were resistant to pemetrexed and C6 ceramide (Fig. 4F and G). More importantly, C6 ceramide-induced pemetrexed sensitization effect was abolished in MC3T3-E1 cells (Fig. 4F and G). These results showed the specific cytotoxic effect of pemetrexed in cancer cells, and Akt/mTOR activation status might be the key determinant of C6 ceramide-induced sensitization effect.

4. Discussions

OS is the most common form of primary bone tumor with a high rate of metastasis. Patients with metastatic OS experience a 5-year survival rate as low as 20% [28,29]. The search for more effective anti-OS chemo-drugs is important. Pemetrexed is a novel anti-folate cytotoxic agent, which potently inhibits the activity of TS, DHFR, and GARFT, thereby depleting nucleotide pools to block DNA synthesis [5,6,30]. Clinically, pemetrexed is used for malignant pleural mesothelioma patients [5–7]. It is also applied as first-line, second-line, and maintenance therapy in the treatment of non-small cell lung cancer [7]. Ataxia telangiectasia mutated (ATM)-p53 signaling activation [21,31] as well as cyclin-A/cyclin-dependent kinase (CDK-2) activation [32] may be the mechanism involved in pemetrexed-induced apoptosis. Further, sustained Erk activation [32] and AMP-activated protein kinase (AMPK) activation might also contribute to pemetrexed-induced cytotoxicity [33].

In pre-clinical studies, pemetrexed displayed broad anti-tumor activity against breast, pancreatic, lung, and colon cancer cell lines [5,6,30]. However, pemetrexed only displayed lower efficacy against osteosarcoma cell lines, its efficiency was not as good as methotrexate [9]. The results from a phase II clinical study showed that pemetrexed as second-line treatment to advanced or metastatic OS failed to reach minimal response expectation [34], although a third of the patients with relapsed OS did survive for at least 1 year [34]. Thus, new strategies should be designed to

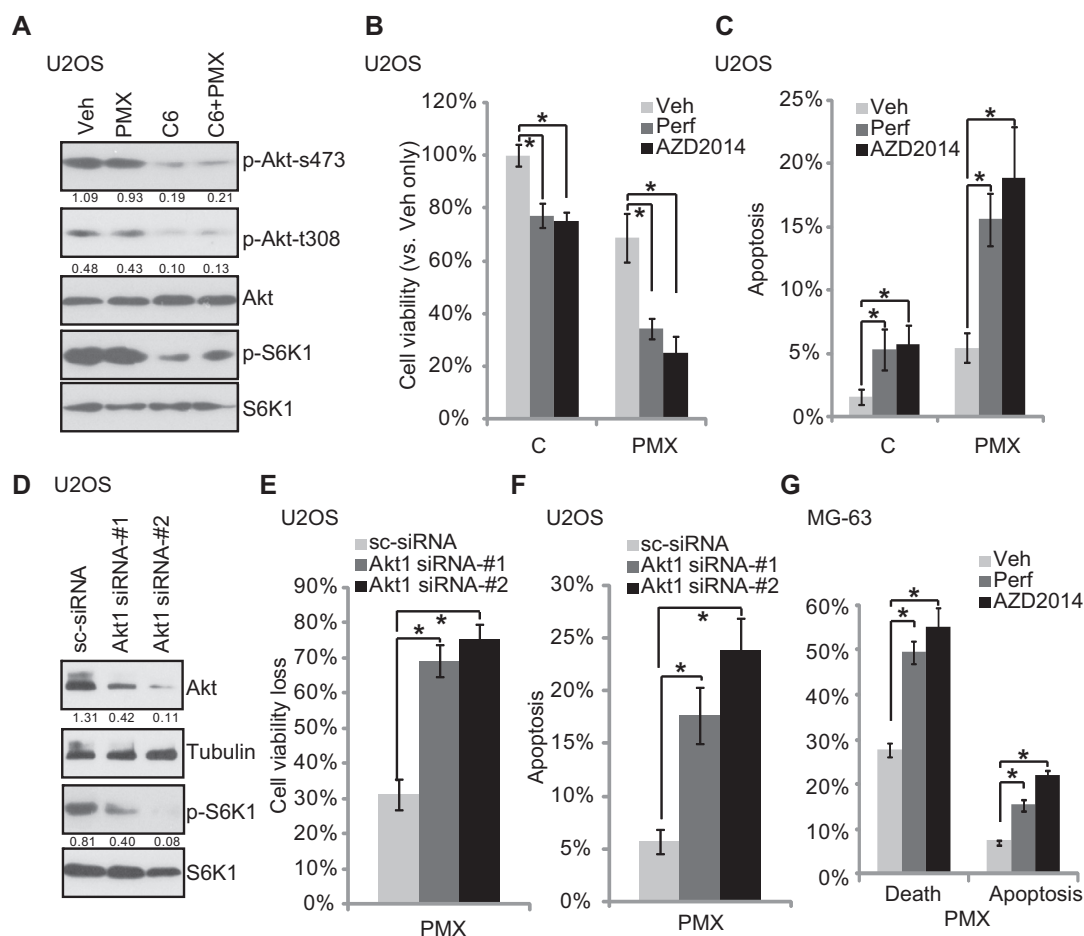


Fig. 3. C6 ceramide in-activates Akt/mTOR to sensitize pemetrexed in OS cells. U2OS cells were treated with pemetrexed (PMX, 1 μ M) and/or C6 ceramide (C6, 5 μ g/ml) for 24 h, phospho-/regular Akt and S6K1 were tested by Western blots (A). U2OS cells were pretreated with perifosine (Perf, 5 μ M) or AZD2014 (100 nM) for 1 h, followed with pemetrexed (1 μ M) stimulation, cell viability and cell apoptosis were analyzed by MTT assay (B) and FACS assay (C), respectively. U2OS cells were transfected with scramble siRNA (200 nM) or Akt1 siRNA (#1 or #2, 200 nM each) for 48 h, indicated proteins were tested (D). Pemetrexed (PMX, 1 μ M) were added to U2OS cells with above siRNAs, cell viability (E) and cell apoptosis (F) were analyzed. MG-63 cells were pretreated with perifosine (Perf, 5 μ M) or AZD2014 (100 nM) for 1 h, followed with pemetrexed (PMX, 1 μ M) stimulation, cell death (trypan blue staining, 72 h) and cell apoptosis (Annexin V FACS, 48 h) were analyzed (G). Each experiment was performed in triplicate and was repeated at least four times. "Veh" stands for 0.5% DMSO. "C" stands for no-pemetrexed treatment, * p < 0.05.

increase the therapeutic efficacy of pemetrexed. A number of studies have studied the potential of C6 ceramide to augment the anti-tumor efficiency of known chemotherapeutic agents (i.e. doxorubicin, taxol and HDACi), and results showed that C6 ceramide could be useful as a very efficient adjunct to conventional chemotherapeutics [14,15,35]. Here we report that co-administration of pemetrexed and ceramide (C6) resulted in a striking increase in cytotoxicity and apoptosis in human OS cell lines.

The Akt/mTOR signaling is important in regulating key processes for cancer cell progression, including protein translation, growth, metabolism, apoptosis resistance and cell survival [36,37]. This pathway is frequently over-expressed and/or over-activated in OS (see Fig. 4), and is an important drug target in OS therapy [36,37]. In this study, we found that Akt/mTOR inhibition by pharmacological inhibitors or by siRNA-knockdown dramatically enhanced pemetrexed-induced cytotoxicity in cultured osteosarcoma cells, indicating that Akt/mTOR signaling is an important pemetrexed resistance factor in OS cells. The role of pemetrexed on Akt phosphorylation has not been consistent in different cancer cell lines. Li et al., demonstrated that pemetrexed itself induced phosphorylated Akt through EGFR-mediated signalings in human non-small cell lung cancer cells [38]. While other reported that pemetrexed decreased Akt phosphorylation in differ-

ent cancer cells [39]. Our data showed that phosphorylation of Akt and its downstream S6K1 was not affected by pemetrexed alone. Akt could be negatively regulated by serine/threonine phosphatase (PP), and ceramide was shown to activate phosphatase 1 (PP1) and phosphatase 2A (PP2A) to de-phosphorylate Akt [40–42], this could explain why we observed Akt in-activation by C6 ceramide in OS cells. In summary, we conclude that C6 ceramide sensitizes pemetrexed-induced apoptosis and cytotoxicity in cultured OS cells probably through Akt–mTOR in-activation.

Conflict of interest

All authors proclaim that there do not exist any financial and personal relationships with other people or organizations that could potentially and inappropriately influence (bias) their work and conclusions.

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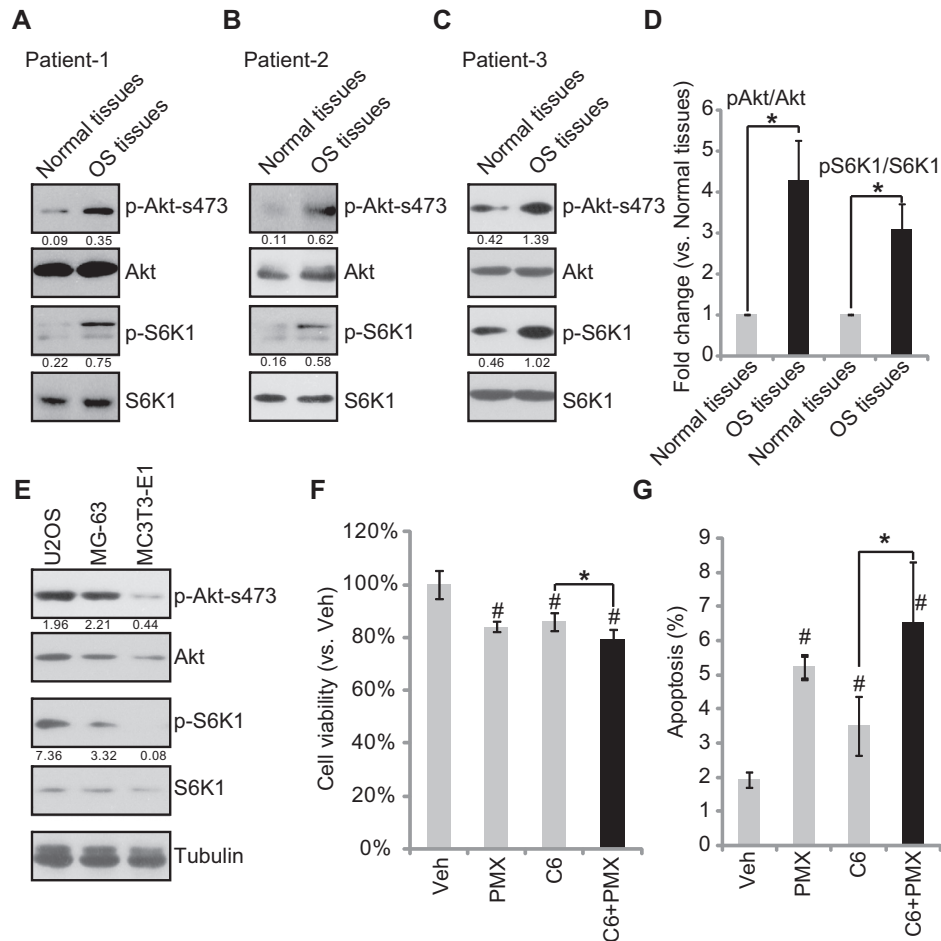


Fig. 4. Over-activation of Akt–mTOR signaling in human OS tissues. Phospho-/regular-Akt and S6K1 in three human OS tissues and corresponding surrounding normal osteo-tissues were tested by Western blots (A–C). Akt/S6K1 phosphorylation was quantified (D). Phospho-/regular-Akt and S6K1 in U2OS cells, MG-63 cells or MC3T3-E1 cells were tested by Western blots (E). MC3T3-E1 cells were treated with pemetrexed (PMX, 1 μ M) and/or C6 ceramide (C6, 5 μ g/ml) for 72 h, cell viability (F, 72 h) and apoptosis (G, 48 h) were tested. “Veh” stands for 0.5% DMSO. # p < 0.05 vs. “Veh” (F and G). * p < 0.05.

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